

Cyclooxygenase-2 inhibitors modulate skin aging in a catalytic activity-independent manner

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Abbreviations: COX-2, cyclooxygenase-2; NSAID, non-steroidal anti-inflammatory drug

Abstract

It has been proposed that the pro-inflammatory catalytic activity of cyclooxygenase-2 (COX-2) plays a key role in the aging process. However, it remains unclear whether the COX-2 activity is a causal factor for aging and whether COX-2 inhibitors could prevent aging. We here examined the effect of COX-2 inhibitors on aging in the intrinsic skin aging model of hairless mice. We observed that among two selective COX-2 inhibitors and one non-selective COX inhibitor studied, only NS-398 inhibited skin aging, while celecoxib and aspirin accelerated skin aging. In addition, NS-398 reduced the expression of p53 and p16, whereas celecoxib and aspirin enhanced their expression. We also found that the aging-modulating

effect of the inhibitors is closely associated with the expression of type I procollagen and caveolin-1. These results suggest that pro-inflammatory catalytic activity of COX-2 is not a causal factor for aging at least in skin and that COX-2 inhibitors might modulate skin aging by regulating the expression of type I procollagen and caveolin-1.

Keywords: caveolin-1; cyclooxygenase inhibitors; procollagen; skin aging

Introduction

Prostaglandin endoperoxide synthase (PTGS), also called cyclooxygenase (COX), is responsible for the conversion of arachidonic acid to prostaglandin H₂ (PGH₂), which is the common precursor for the biosynthesis of various prostanoids including prostaglandin D₂ (PGD₂), prostaglandin E₂ (PGE₂), prostaglandin I₂ (PGI₂) and thromboxane A₂ (TXA₂). Among them, PGE₂ and PGI₂ are known to be important inflammatory mediators. There are two isozymes of COX, COX-1 and COX-2. In general, COX-1 is a constitutive type, whereas COX-2 is an inducible type whose expression is increased by various stimuli (Smith *et al.*, 1996; Funk, 2001; Rouzer and Marnett, 2008; Park and Kwon, 2011).

Most non-steroidal anti-inflammatory drugs (NSAIDs) are COX inhibitors, which occupy the active site of COX to inhibit the enzyme's catalytic activity. Traditionally used NSAIDs including aspirin, ibuprofen and indomethacin inhibit the catalytic activity of both COX-1 and COX-2 non-selectively. In contrast, recently developed NSAIDs such as NS-398, celecoxib or rofecoxib inhibit the catalytic activity of COX-2 selectively (Flower, 2003).

While underlying mechanisms of aging have not been fully elucidated, it has been proposed that the pro-inflammatory catalytic activity of COX-2 plays a crucial role in the aging process. Reactive oxygen species (ROS) generated in the process of normal metabolism or inflammation have been suggested to activate the redox-sensitive transcription factor NF- κ B inducing the expression of pro-inflammatory genes such as COX-2, which in turn stabilizes

chronic inflammatory status by producing ROS to cause tissue damage and aging (Chung *et al.*, 2009).

If the pro-inflammatory catalytic activity of COX-2 plays a causal role for aging, COX-2 inhibitors will have to inhibit the aging process. However, it has not yet been determined whether the catalytic activity of COX-2 is involved in the aging process and whether COX-2 inhibitors prevent aging. Actually, expression levels of COX-2 are observed to increase in heart and kidney of aged rats (Kim *et al.*, 2000; Kim *et al.*, 2001), but not in brain of aged rats and endothelial cells of aged humans (Baek *et al.*, 2001; Sanguino *et al.*, 2004; Donato *et al.*, 2008). In addition, non-selective COX inhibitors such as acetylsalicylic acid, salicylic acid and indomethacin do not affect or rather shorten the life span in *Drosophila* (Massie *et al.*, 1985). A recent report shows that celecoxib, a selective COX-2 inhibitor, extends the life span in *C. elegans*, which is mediated by a COX-2 catalytic activity-independent mechanism (Ching *et al.*, 2011).

In the present study, to elucidate whether the catalytic activity of COX-2 is a causal factor for aging, we analyzed the effect of COX-2 inhibitors on aging in the intrinsic skin aging model of hairless mice. Our data shows that NS-398 (a selective COX-2 inhibitor) inhibits skin aging, while celecoxib (a selective COX-2 inhibitor) and aspirin (a non-selective COX inhibitor) accelerate skin aging. In addition, the aging-modulating effect of the inhibitors was associated with the expression of p53, p16, type I procollagen and caveolin-1.

Results

COX-2 inhibitors modulate intrinsic skin aging in a catalytic activity-independent manner

To elucidate whether the COX-2 catalytic activity is a causal factor for intrinsic skin aging, we analyzed the effect of two selective COX-2 inhibitors (NS-398 and celecoxib) and one non-selective COX inhibitor (aspirin) on intrinsic skin aging in SKH-1 hairless mice. The IC₅₀ values of NS-398, celecoxib and aspirin had been reported for recombinant human or ovine COX-1 and COX-2 (Barnett *et al.*, 1994; Johnson *et al.*, 1995). In addition, we had previously established that around 10-fold higher concentrations of IC₅₀ of NS-398 and celecoxib (20 μM and 0.5 μM, respectively) as well as IC₅₀ of aspirin (1.0 mM) inhibited the COX-2 catalytic activity significantly without cellular toxicity in human dermal fibroblasts (HDFs). We had used IC₅₀ for aspirin because 10-fold higher concentration caused acute cellular toxicity (Kim *et al.*, 2008). In the present study, we

used 5-fold and 50-fold higher concentrations for each drug than the concentrations used in HDFs to inhibit the COX-2 catalytic activity sufficiently in *in vivo* skin (Table 1).

Intrinsic skin aging is characterized by thinning, sagging and wrinkling of the skin (Rittié and Fisher, 2002). Thus, we applied the inhibitors everyday onto the right and left side of dorsal skin of the mice for 12 weeks and measured their skin fold thickness by using a caliper. The data showed that skin fold thickness was decreased by ~20% after 12 weeks when compared to 0 weeks under the treatment of the vehicle (ethanol: propylene glycol = 7:3), which was significantly prevented by the treatment of NS-398 (Figure 1A). In contrast, celecoxib and aspirin further decreased the skin fold thickness as compared to the vehicle (Figures 1B and 1C).

Skin is composed of two layers, the epidermis and the dermis, whose thickness has been known to decrease in the intrinsic skin aging (Varani *et al.*, 2000). Therefore, we obtained skin tissues at the end of the drug treatment for 12 weeks to measure epidermal thickness. It was observed that the treatment of NS-398 increased epidermal thickness (Figures 2A and 2D), whereas the treatment of celecoxib and aspirin decreased epidermal thickness as compared to the treatment of the vehicle (Figures 2B-2D). These results show that NS-398 inhibits the aging-associated thinning of the skin while celecoxib and aspirin accelerate it.

We then analyzed the effect of the inhibitors on wrinkling of the skin by using skin replica. The data showed that the treatment of NS-398 greatly reduced average wrinkle depth as compared to the treatment of the vehicle (Figures 3A and 3D). On the contrary, celecoxib treatment significantly increased average wrinkle depth as compared to the treatment of the vehicle (Figures 3B and 3D). In the case of aspirin, we observed that the average wrinkle depth was prominently increased by the treatment of 50 mM aspirin but not by the treatment of 5 mM aspirin (Figures 3C and 3D). The maximum wrinkle depth and average wrinkle area were also measured in the same replica and the data showed exactly the same tendency with the average wrinkle depth (data not shown). These results indicate that NS-398 inhibits

Table 1. IC₅₀ values of COX-2 inhibitors and used concentrations

Inhibitors	IC ₅₀ for COX-1	IC ₅₀ for COX-2	Used doses in HDFs	Used doses in mice
NS-398	75 μM	1.77 μM	20 μM	0.1 / 1.0 mM
Celecoxib	15 μM	0.04 μM	0.5 μM	2.5 / 25 μM
Aspirin	0.75 mM	1.25 mM	1.0 mM	5 / 50 mM

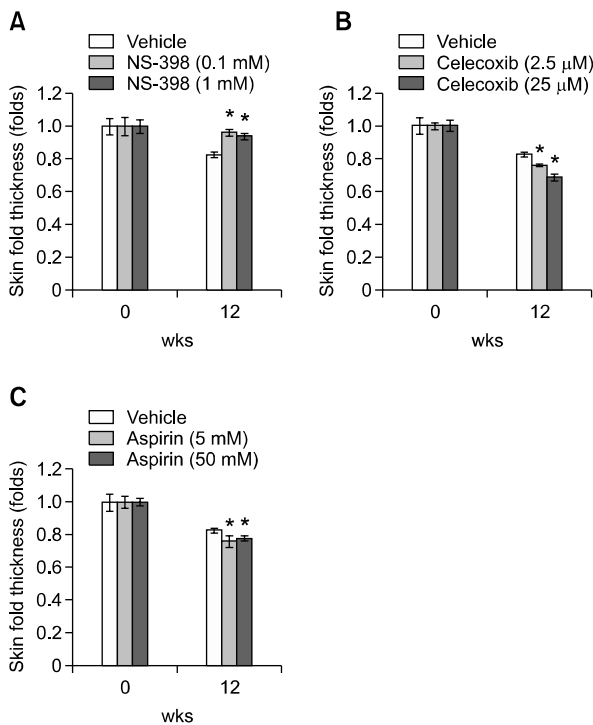


Figure 1. NS-398 increases but celecoxib and aspirin decrease skin fold thickness in hairless mice. NS-398 (A), celecoxib (B) and aspirin (C) were treated to the right and left side of dorsal skin of mice for 12 weeks. Skin fold thickness was measured by using a caliper. Data represent means \pm S.E. ($n = 5$). * $P < 0.05$; compared with the corresponding vehicle-treated group.

the aging-associated wrinkling of the skin, whereas celecoxib and high dose of aspirin accelerate it.

Collectively, these results demonstrate that NS-398 inhibits the intrinsic skin aging while celecoxib and aspirin accelerate it, suggesting that the catalytic activity of COX-2 does not mediate intrinsic skin aging and that COX-2 inhibitors modulate intrinsic skin aging through a catalytic activity-independent mechanism.

The aging-modulating effect of COX-2 inhibitors is associated with p53 and p16 expression

It is widely accepted that diverse stimuli inducing cellular senescence ultimately activate either or both of p53 and p16/pRB pathway. Although it has not been well established that these pathways are also critical channels for individual aging, accumulating evidence indicates that the p53 and p16/pRB pathways are common pathways in individual aging, too (Campisi, 2005).

Therefore, to support the aging-modulating effect of the inhibitors on the skin aging at molecular levels, we examined expression levels of p53 and p16 under the treatment of the inhibitors. The

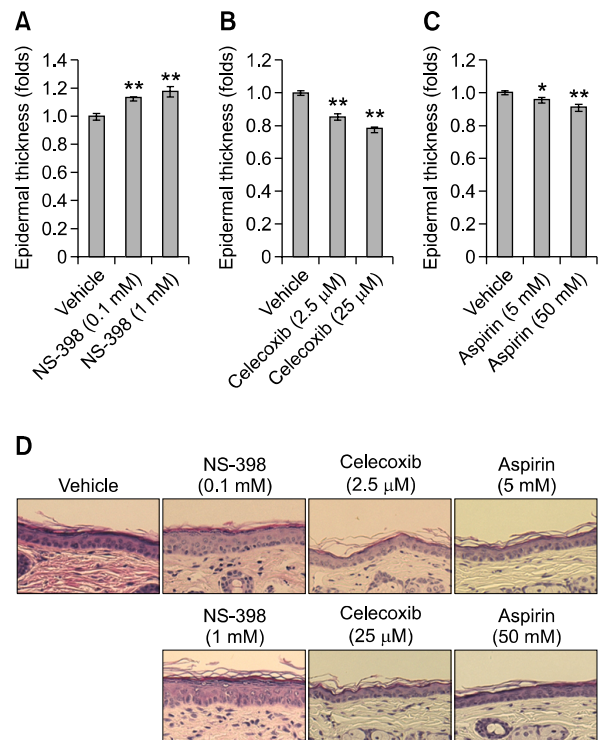


Figure 2. NS-398 increases but celecoxib and aspirin decrease epidermal thickness in hairless mice. NS-398 (A), celecoxib (B) and aspirin (C) were treated to the right and left side of dorsal skin of mice for 12 weeks. Paraffin sections of the skin were stained with H & E and then epidermal thickness was measured. Data represent means \pm S.E. ($n = 10$). * $P < 0.05$ and ** $P < 0.01$; compared with the corresponding vehicle-treated group. (D) Representative images of H & E staining were shown.

western blotting data showed that NS-398 inhibited expression of both p53 and p16 dose-dependently (Figures 4A and 4D), while celecoxib and aspirin enhanced the expression of p53 and p16 dose-dependently as compared to the vehicle (Figures 4B-4D). These results suggest that COX-2 inhibitors modulate the skin aging through both p53 and p16/pRB pathways, and support our conclusion at molecular levels that NS-398 inhibits intrinsic skin aging whereas celecoxib and aspirin accelerate it.

The aging-modulating effect of COX-2 inhibitors is associated with type I procollagen expression

Skin is predominantly composed of type I collagen, which is synthesized in dermal fibroblasts and secreted as the procollagen form. The expression of type I procollagen is greatly reduced in intrinsic skin aging, which is thought to be a crucial factor for degenerative changes such as thinning and wrinkling of the skin (Chung *et al.*, 2001; Rittié and Fisher, 2002; Shoulders and Raines, 2009).

To test the possibility that COX-2 inhibitors might

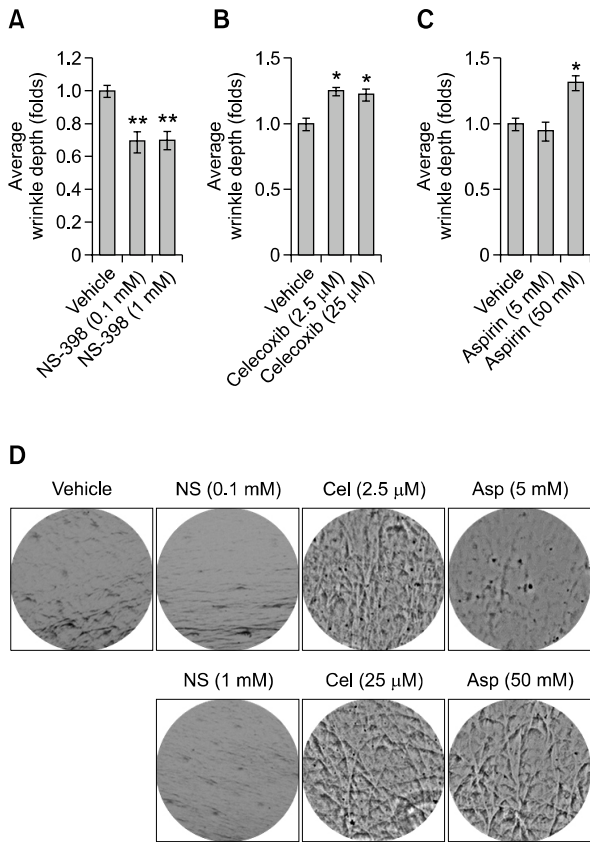


Figure 3. NS-398 decreases but celecoxib and high dose of aspirin increase average wrinkle depth in hairless mice. NS-398 (A), celecoxib (B) and aspirin (C) were treated to the right and left side of dorsal skin of mice for 12 weeks. Skin replicas were obtained and average wrinkle depth was measured. Data represent means \pm S.E. ($n = 10$). * $P < 0.05$ and ** $P < 0.01$; compared with the corresponding vehicle-treated group. (D) Representative images of skin replicas were shown.

regulate the expression of type I procollagen in the skin, we monitored protein levels of type I procollagen under the treatment of the inhibitors. The western blotting data showed that NS-398 markedly increased the amount of type I procollagen dose-dependently (Figures 5A and 5D), whereas celecoxib and aspirin decreased the amount of type I procollagen dose-dependently as compared to the vehicle (Figures 5B-5D). These results demonstrate that the aging-modulating effect of the inhibitors is associated with type I procollagen expression, suggesting that COX-2 inhibitors might modulate intrinsic skin aging via regulation of type I procollagen expression.

The aging-modulating effect of COX-2 inhibitors is associated with caveolin-1 expression

Caveolin-1, a structural component of caveolae in the plasma membrane, has been proposed to be an important mediator for cellular senescence (Cho

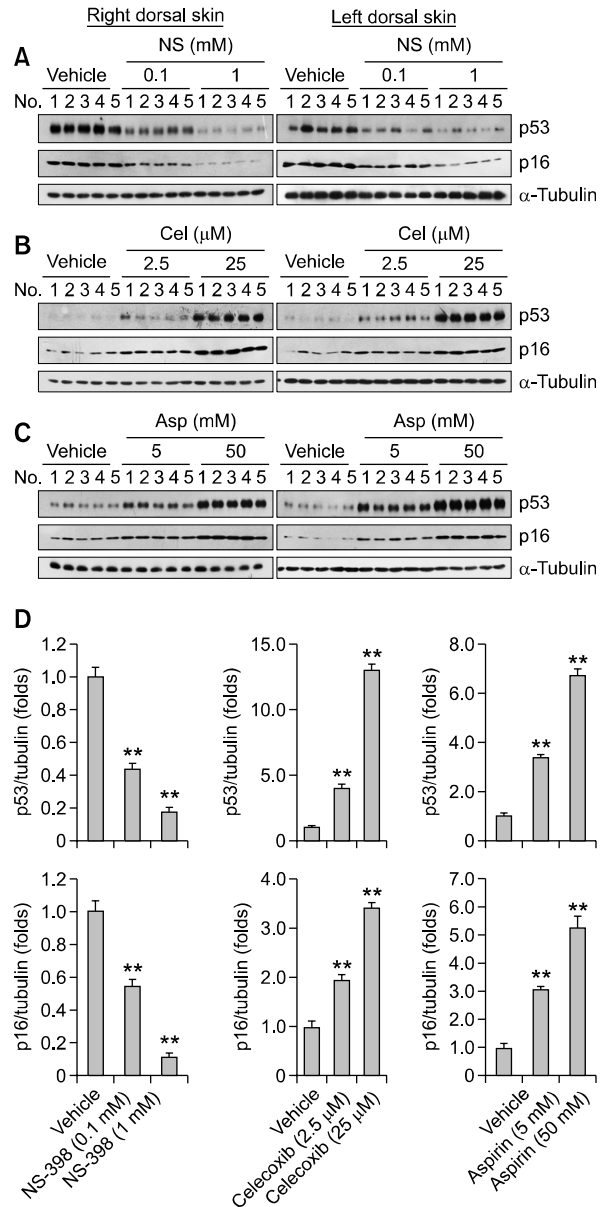


Figure 4. NS-398 decreases but celecoxib and aspirin increase the expression of p53 and p16. NS-398 (A), celecoxib (B) and aspirin (C) were treated to the right and left side of dorsal skin of mice for 12 weeks. Skin lysates were prepared from the indicated numbered mice and western blot analysis was carried out using antibodies against p53 and p16. α -Tubulin was used as a loading control. Exposing times are variable to acquire the best quality of the data. (D) Quantitative analysis for p53 and p16 expression was done by densitometry. Data represent means \pm S.E. ($n = 10$). ** $P < 0.01$; compared with the corresponding vehicle-treated group.

and Park, 2005). In addition, we had previously observed that the treatment of COX-2 inhibitors altered the expression of caveolin-1 in HDFs (Kim *et al.*, 2008).

Therefore, to test the possibility that the inhibitors might regulate caveolin-1 expression in the skin

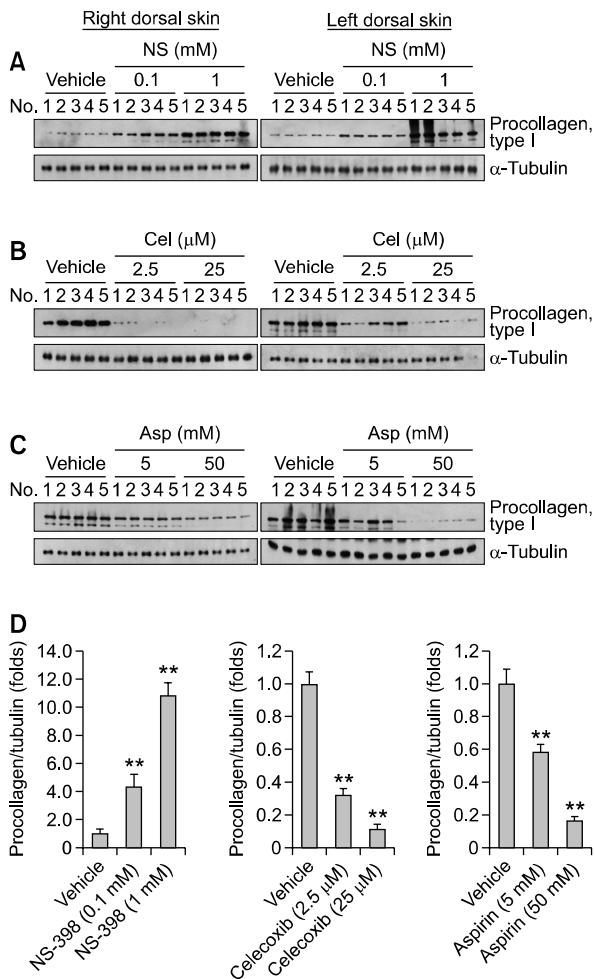


Figure 5. NS-398 increases but celecoxib and aspirin decrease the expression of type I procollagen. NS-398 (A), celecoxib (B) and aspirin (C) were treated to the right and left side of dorsal skin of mice for 12 weeks. Skin lysates were prepared from the indicated numbered mice and western blot analysis was carried out using an antibody for type I procollagen. α -Tubulin was used as a loading control. Exposing times are variable to acquire the best quality of the data. (D) Quantitative analysis for type I procollagen expression was done by densitometry. Data represent means \pm S.E. ($n = 10$). $**P < 0.01$; compared with the corresponding vehicle-treated group.

aging, we monitored expression levels of caveolin-1 under the treatment of the inhibitors. As shown in Figures 6A and 6D, NS-398 decreased the amount of caveolin-1 dose-dependently as compared to the vehicle. On the contrary, celecoxib and aspirin increased the amount of caveolin-1 dose-dependently as compared to the vehicle (Figures 6B-6D). These results demonstrate that the aging-modulating effect of the inhibitors is associated with caveolin-1 expression, suggesting that COX-2 inhibitors might modulate intrinsic skin aging *via* regulation of caveolin-1 expression.

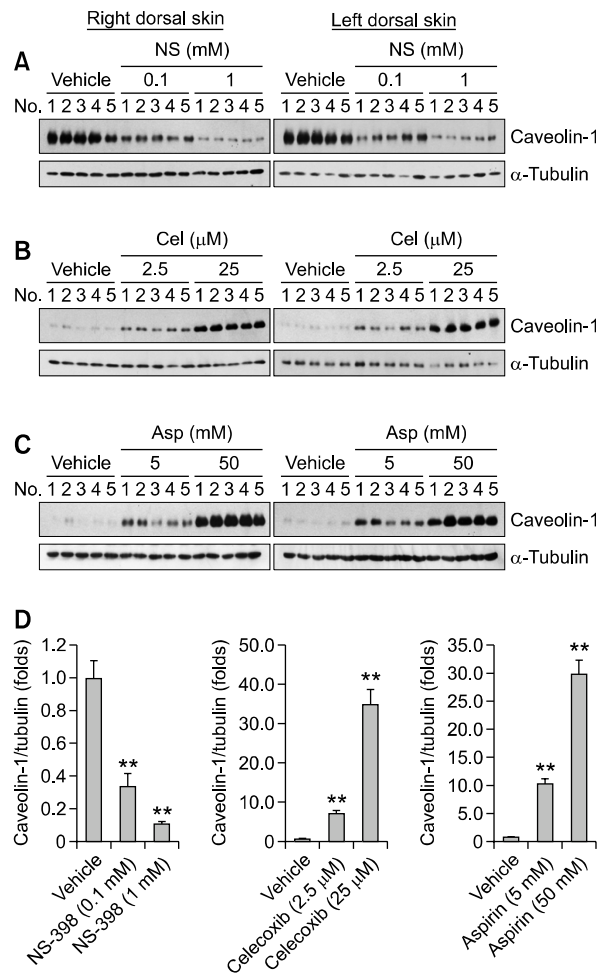


Figure 6. NS-398 decreases but celecoxib and aspirin increase the expression of caveolin-1. NS-398 (A), celecoxib (B) and aspirin (C) were treated to the right and left side of dorsal skin of mice for 12 weeks. Skin lysates were prepared from the indicated numbered mice and western blot analysis was carried out using an anti-caveolin-1 antibody. α -Tubulin was used as a loading control. Exposing times are variable to acquire the best quality of the data. (D) Quantitative analysis for caveolin-1 expression was done by densitometry. Data represent means \pm S.E. ($n = 10$). $**P < 0.01$; compared with the corresponding vehicle-treated group.

Discussion

The pro-inflammatory catalytic activity of cyclooxygenase-2 (COX-2) has been proposed to be a crucial factor for individual aging as well as cellular senescence (Zdanov *et al.*, 2007; Chung *et al.*, 2009). In the present study, we have investigated whether COX-2 inhibitors could prevent aging in the intrinsic skin aging model of hairless mice. Our data showed that among three COX-2 inhibitors studied, only NS-398 inhibits the skin aging while celecoxib and aspirin accelerate it (Figures 1-3), suggesting that the catalytic activity of COX-2 does not mediate aging at least in skin. Consistent with these results,

we had previously observed that among six COX-2 inhibitors studied, only NS-398 inhibited cellular senescence whereas two selective COX-2 inhibitors (celecoxib and nimesulide) and three non-selective COX inhibitors (aspirin, ibuprofen and flurbiprofen) accelerated the senescence in human dermal fibroblasts (HDFs) (Kim *et al.*, 2008).

It is still controversial whether cellular senescence is a source for individual aging. At least in skin, however, senescent fibroblasts have been detected in the dermis and their numbers have been found to increase with age in mice, baboons and humans (Dimri *et al.*, 1995; Herbig *et al.*, 2006; Wang *et al.*, 2009). According to our present and previous studies, the aging-modulating effect of COX-2 inhibitors in *in vivo* skin is consistent with the senescence-modulating effect of the inhibitors in dermal fibroblasts (Figures 1-3; Kim *et al.*, 2008). These findings support the idea that cellular senescence is a source for individual aging, and suggest that modulation of cellular senescence might be a good strategy to modulate individual aging.

Cellular senescence can be induced by diverse stimuli including dysfunctional telomeres, DNA damage, oncogenes and others, which ultimately activate either or both of the p53 and p16/pRb pathways. In general, the activity of p53 increases in the course of cellular senescence with or without an elevation of p53 expression (Atadja *et al.*, 1995; Kulju and Lehman, 1995). The p16 expression is usually enhanced in cellular senescence (Alcorta *et al.*, 1996). Compatible with these results, expression levels of p53 increases in brain of aged rats (Dorszewska and Adamczewska-Goncerzewicz, 2004). In the case of heart, kidney, liver and lung, the total protein amount of p53 is not changed but the amount of acetylated p53 increases in aged rats (Braidly *et al.*, 2011). On the other hand, the p16 expression increases in most tissues of aged mice and rats (Krishnamurthy *et al.*, 2004). These studies suggest that the p53 and p16/pRb pathways are ultimate channels for individual aging, too. We observed here that the expression of p53 and p16 was changed by the treatment of COX-2 inhibitors, which was in agreement with the aging-modulating effect of the inhibitors (Figure 4). These data suggest that COX-2 inhibitors modulate skin aging by affecting both p53 and p16/pRb pathways and strongly support our conclusion at molecular levels that NS-398 inhibits intrinsic skin aging whereas celecoxib and aspirin accelerate it.

According to our data, aspirin did not alter the average wrinkle depth at the concentration of 5 mM, whereas 50 mM of aspirin markedly increased the average wrinkle depth (Figure 3C). However, skin

fold thickness and epidermal thickness were decreased by the treatment of both 5 mM and 50 mM of aspirin, showing that aspirin treatment caused skin thinning at both concentrations (Figures 2C and 3C). In addition, expression levels of p53 and p16 were also elevated by the treatment of 5 mM of aspirin, verifying at molecular levels that 5 mM of aspirin accelerated the skin aging (Figure 4C). For the reason of this discrepancy, we assume that the thinning of skin precedes and wrinkle formation appears later in the course of intrinsic skin aging.

It is now uncertain how COX-2 inhibitors modulate the skin aging. Nonetheless, we have here suggested that COX-2 inhibitors modulate the skin aging by regulation of type I procollagen and caveolin-1 expression. We observed that NS-398 increased the type I procollagen expression while celecoxib and aspirin decreased it (Figure 5). Compatible with these results, it has been reported that NS-398 enhances type I procollagen expression in HDFs and promotes fibrosis in rat muscle (Han *et al.*, 2004; Shen *et al.*, 2005). In addition, celecoxib reduces type I collagen expression in mouse mammary gland and rat liver (Paik *et al.*, 2009; Lyons *et al.*, 2011). Aspirin has been reported to reduce collagen biosynthesis in HDFs (Surazynski *et al.*, 2004). Since type I collagen is the major constituent of the extracellular matrix (ECM) of skin and the reduction of its content is associated with degenerative changes as seen in the intrinsic skin aging (Rittié and Fisher, 2002), our results suggest that COX-2 inhibitors might modulate the skin aging through regulation of type I procollagen expression. In addition, since type I collagen is the most abundant protein in the ECM of most tissues, there is a possibility that COX-2 inhibitors modulate aging not only in skin but also in other tissues. Further studies are required to elucidate the above possibility and the mechanism by which COX-2 inhibitors regulate the expression of type I procollagen.

Caveolae are flask-shaped vesicular invaginations in the plasma membrane and play central roles in receptor-mediated endocytosis. Caveolin-1 is the main component of caveolae, which has been proposed to be a key determinant for aging (Zou *et al.*, 2011). Actually, the expression of caveolin-1 is enhanced not only in cellular senescence but also in brain, heart, kidney, lung and spleen in aged rats (Park *et al.*, 2000). In addition, overexpression of caveolin-1 reduces cellular life span in mouse fibroblasts and siRNAs for caveolin-1 triggers reinitiation of DNA synthesis in senescent HDFs (Volonte *et al.*, 2002; Cho *et al.*, 2003). It appears that this pro-aging effect of caveolin-1 is attributable to its capability to interact with other aging-modulating

molecules in caveolae. Under the treatment of hydrogen peroxide, caveolin-1 interacts with the antioxidant enzyme thioredoxin reductase 1 (TrxR1) to inhibit its enzymatic activity, which activates the p53/p21 pathway and premature senescence in NIH3T3 cells (Volonte and Galbiati, 2009). In addition, caveolin-1 interacts with the ubiquitin ligase Mdm-2 to sequester it to the caveolar membrane, resulting in the activation of p53 and premature senescence in human and mouse fibroblasts (Bartholomew *et al.*, 2009). We here reported that the aging-modulating effect of COX-2 inhibitors is closely associated with caveolin-1 expression levels (Figure 6), suggesting that the inhibitors might modulate the skin aging by regulation of caveolin-1 expression. Consistent with these findings, we have previously reported that among three selective COX-2 inhibitors studied, NS-398 inhibited caveolin-1 expression whereas the other inhibitors (celecoxib and nimesulide) enhanced the expression, which was closely associated with the senescence-modulating effect of the inhibitors in HDFs (Kim *et al.*, 2008). Further studies are required to elucidate the mechanism by which COX-2 inhibitors regulate the expression of caveolin-1.

In conclusion, the results of this study suggest that the catalytic activity of COX-2 is not a causal factor for intrinsic skin aging as evidenced by acceleration of the skin aging by celecoxib and aspirin among three COX-2 inhibitors studied. However, it still remains to be elucidated whether COX-2 catalytic activity is involved in the intrinsic aging in other tissues. Therefore, it is necessary to investigate continuously the function of COX-2 and effects of COX-2 inhibitors at individual levels.

Methods

Materials

NS-398 and aspirin were purchased from Cayman Chemicals (Ann Arbor, MI). Celecoxib was a generous gift from Dr. S.V. Yim (Kyung Hee University, Korea). Drugs were diluted in a solution of ethanol and propylene glycol (7:3).

Animals and drug treatment

All procedures were approved by the Institutional Animal Care and Use Committee of the Kangwon National University (KIACUC-09-0017). Female SKH-1 hairless mice at 6 weeks of age were obtained from Orient Bio Inc. (Seongnam, Korea) and were acclimated in our facility for a week before the experiments. Five mice were allocated to each group and were treated with 100 μ l of drugs every day for 12 weeks on the right and left side of their dorsal skin.

Measurement of skin fold thickness

Midline skin was manually pinched upward at the neck and base of the tail. Then, skin fold thickness was measured midway between the neck and hip by using a caliper (PEACOCK, Ozaki MFG Co., Tokyo, Japan) as described previously (Kim *et al.*, 2005).

Measurement of epidermal thickness

Skin samples were fixed in 10% formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H & E), and epidermal thickness was measured by using the Labworks software (PerkinElmer Inc., Waltham, MA).

Measurement of wrinkle grade

Skin replicas were obtained by applying silicon rubbers (Cuderm Corp., Dallas, TX) onto the murine skin. The replicas were photographed and wrinkles were analyzed by the skin visiometer SV600 (Courage-Khazaka Electronic GmbH, Köln, Germany).

Western blot analysis

Skin samples were frozen and ground in liquid nitrogen and then homogenized in RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, 1% Tween-20, 1% sodium deoxycholate and 0.1% SDS) with 0.5 mM EDTA, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 μ g/ml pepstatin. The lysates were resolved in SDS-PAGE and transferred to nitrocellulose membranes, and probed with appropriate antibodies. The immunoreactive protein complexes were detected by enhanced chemiluminescence (Amersham Bioscience, Boston, MA). Mouse monoclonal antibodies for p53 (Ab-6), p16 (sc-74401) and caveolin-1 (#601406) were purchased from Oncogene Science (Cambridge, MA), Santa Cruz Biotechnology (Danvers, MA) and BD Biosciences (San Jose, CA), respectively. A rabbit polyclonal antibody specific for type I procollagen (BCO-A3001) was purchased from BioCol Inc. (Lake Forest, CA).

Statistical analysis

Comparison between two groups was tested by the student's *t* test using the GraphPad Prism program (GraphPad Software). The difference between two groups was statistically significant if *P* values were less than 0.05.

Acknowledgements

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